Identification of Natural Antimicrobial Substances in Red Muscadine Juice against *Cronobacter sakazakii*

T.J. Kim, W.L. Weng, J.L. Silva, Y.S. Jung, and D. Marshall

ABSTRACT: Red muscadine (*Vitis rotundifolia* Michx.) juices with natural organic, phenolic acids and polyphenol compounds were tested against *Cronobacter sakazakii*. The concentration of total phenolic compounds of commercial baby juices ranged from 176.7 to 347.7 mg/mL. Commercial baby juices showed poor antimicrobial activity, reducing less than 1-log of *C. sakazakii* in juice samples for 2 h at 37 °C. Red muscadine juices, regardless of processing methods (filtration, pasteurization, and sterilization), achieved a 6-log reduction of *C. sakazakii* in the same time period (2 h). The mixture of synthetic organic acids (malic and tartaric acids) and polyphenolic acid (tannic acid) showed strong antimicrobial activity against *C. sakazakii*. Among synthetic organic acids, tannic acid was undetected in commercial baby juices. Tannic acid showed the highest antimicrobial activity (1.4- to 3.8-log reduction) against *C. sakazakii*, while malic and tartaric acids showed less than 0.5-log reduction. These results suggest that red muscadine juice could be utilized as a natural antimicrobial in baby food formulations to inhibit *C. sakazakii*.

Keywords: *Cronobacter sakazakii*, malic acid, red muscadine juice, tannic acid, tartaric acid

Introduction

Cronobacter sakazakii is considered an emerging opportunistic pathogen, causing rare but life-threatening meningitis, bacteremia, and necrotizing enterocolitis in infants (Nazarowec-White and Farber 1997; Lai 2001). Outbreaks of *C. sakazakii* have been associated with the ingestion of contaminated (less than 1 cell per 100 g) infant formula (Iversen and Forsythe 2003). *Cronobacter sakazakii* inoculated on infant cereals rapidly grew in infant rice or oatmeal cereals reconstituted with water, apple juice, milk, or infant formula (Richards and others 2005; Lin and Beuchat 2007). Many strains of *C. sakazakii* were shown to have acid resistance in acidified tryptic soy broth (pH 3.5) and fruit juices with low pH values (3.6 to 3.9) (Kim and Beuchat 2005; Edelson-Mammel and others 2006; Lin and Beuchat 2007). In addition, *C. sakazakii* has shown various levels of resistance to disinfectants, which are used on surfaces for formula preparation areas in hospitals, food service kitchens, and day-care centers (Beuchat and others 2009). Due to *C. sakazakii* resistance to various organic acids and disinfectants, it is necessary to find novel anti-*C. sakazakii* ingredients which could be formulated in baby foods.

Muscadine grapes (*Vitis rotundifolia*) have the ability to tolerate hot, humid climates, and resist Pierce's disease. They are the predominant grape species cultivated in the southeastern United States (Chen and others 2001). Recently, interest in the health benefits of muscadines has increased due to their high phenolic content (Pastrana-Bonilla and others 2003; Yi and others 2005), excellent antioxidant capacity (Pastrana-Bonilla and others 2003; Lee and Talcott 2004), anticancer property (Yi and others 2005; Mertens-Talcott and others 2006), and anti-inflammatory activity (Greenspan and others 2005; Bralley and others 2007). We hypothesize that red muscadine juice, rich in natural organic acids (tartaric and malic acids), phenolic acids (tannic and gallic acids), and phenolic compounds and their derivatives may have antimicrobial activity against *C. sakazakii* (Chung and others 1998; Cowan 1999; Akiyama and others 2001; Kim and others 2008) and therefore could be a good candidate for liquid baby products that potentially carry the pathogen. The objectives of this research were to investigate the antimicrobial activity of raw and processed red muscadine juice on *C. sakazakii* and to identify these antimicrobial substances.

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Materials and Methods

Chemicals and reagents

Folin & Ciocalteu's phenol reagent, sodium carbonate and standards of gallic acid (90% purity), (+)-catechin (95% purity), (−)-epicatechin (90% purity), ellagic acid (95% purity), myricetin (85% purity), quercetin (98% purity), trans-resveratrol (95% purity), L-malic acid, and tannic acid were purchased from Sigma-Aldrich Inc. (St. Louis, Mo., U.S.A.). Tartaric acid, acetic acid, hydrochloric acid, sulfuric acid, acetonitrile, methanol, and high-performance liquid chromatography (HPLC) grade water were purchased from Fisher Scientific Inc. (Pittsburgh, Pa., U.S.A.).

Preparation of juice samples

Red muscadine juice (hot-pressed) donated by the Mississippi Agricultural and Forestry Experiment Station (MAFES), Mississippi State Univ. was processed from purple grape cultivar (Noble). The samples of red muscadine juice were divided into 30 experimental observations in 50 mL sterile centrifuge tubes (Fisher Scientific Inc.) and stored at −20 °C until use or further treatments.

doi: 10.1111/j.1750-3841.2010.01531.x

Red muscadine juice was subjected to conventional pasteurization (90 °C, 1 min) (Montville and others 2005; Cortés and others 2008) with constant stirring in an open steam kettle in the Ammerman-Hearnsberger Processing Laboratory, Dept. of Food Science, Nutrition and Health Promotion, Mississippi State Univ., and filled into sterilized Pyrex media bottles (Corning Inc., Corning, N.Y., U.S.A.), and sealed hermetically. After cooling in a refrigerator overnight, the bottled, pasteurized muscadine juices were stored at −20 °C until used. Hot sterile juice samples were prepared by autoclaving (121 °C, 103.4 kPa) the thawed fresh juice in a screw-capped vial (National Scientific Co., Rockwood, Tenn., U.S.A.) for 15 min. Cold filtered juice samples were made by filtering thawed fresh juices through a 0.20 μm syringe filter (Millipore, Bedford, Mass., U.S.A.). Both sterilized and filtered red muscadine juice samples were stored in a refrigerator until use.

Three types of commercial baby juices were purchased from local stores (Starkville, Miss., U.S.A.) and used as reference juice samples in this study. The white grape and apple grape juices were made from juice concentrate, and the mixed berry juice was prepared from juice and puree concentrates. All these products were labeled as preservative free.

Determination of total phenolics

Juice samples were analyzed for total phenolics according to the Folin–Ciocalteu procedure (Waterhouse 2001). Properly diluted juice samples with distilled water were mixed with Folin–Ciocalteu reagent and sodium carbonate. The samples were allowed to develop an intense blue color, which occurred in approximately 60 min. Absorption at 765 nm was measured in a Spectronic Genesys 5 UV-vis spectrophotometer (Fisher Scientific Inc.). The total phenolics were expressed as milligrams of gallic acid equivalents per milliliter of juice.

Determination of the content of soluble solids and pH

The concentration of soluble solids was measured using a Bausch & Lomb Refractometer 33.46.10 (Bauch & Lomb Inc., Rochester, N.Y., U.S.A.). Temperature was maintained at 20 °C with water circulating through the refractometer during the measurement. After calibration with deionized water before each reading, one drop (about 300 μL) of juice sample was placed on the glass prism. The readings were recorded and expressed as °Brix.

The pH values of juices were measured at room temperature (about 25 °C) with a Corning Pinnacle 530 pH meter (Switzerland).

Identification and quantification of major polyphenolic and organic compounds

Reversed-phase HPLC was used to separate and quantify major phenolics in juices. Juice samples were mixed with 6 N HCl at 1:10 ratio (v/v) and placed in a water bath (95 ± 5 °C) for 1 h to remove carbohydrates attached to the phenolic compounds to make aglycons (Kim and others 2008). After cooling to room temperature (about 15 min), each supernatant of the acid hydrolyzed samples was filtered through a 0.45 μm syringe filter (Millipore, Bedford, Mass., U.S.A.) and injected (25 μL) into a Gemini C18, 250 × 4.6 mm column (Phenomenex Inc., Torrance, Calif., U.S.A.) in an Agilent HPLC 1100 series (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.) equipped with a diode array detector. The 2 mobile phases were solvent A: methanol/acetic acid/water (10:2:88, v/v/v), and solvent B: acetonitrile. A linear gradient for phenolics separation was used as follows: at 0 min, 95% solvent A, 5% solvent B; at 1 min, 90% solvent A, 10% solvent B; at 30 min, 30% solvent A, 70% solvent B; at 31 min, 90% solvent A, 10% solvent B; at 32 min, 95% solvent A, 5% solvent B with 5 min post run. The flow rate was 1 mL/min. Individual phenolics were detected at 260 nm. Major organic acids were also similarly separated and quantified by using HPLC. Juice samples were centrifuged at 12000 rpm (1700 *g*) for 5 min, and each supernatant was filtered and injected into a HPLC system as described above. Mobile phase was 0.01 N H_2SO_4 with a flow rate of 1 mL/min. Individual organic acids were detected at 215 nm. Peaks for phenolic compounds and organic acids were integrated and analyzed using the ChemStation software (Agilent Technologies Inc.). Individual compound was identified and quantified, based on the retention time and peak area of each standard, respectively.

Antimicrobial activity of red muscadine and commercial baby juices

Two strains of *C. sakazakii* Fec39 and MSDH were used in this study. These strains were clinically isolated by Dr. Ute Römling (Microbiology and Tumorbiology Center, Sweden) (Zogaj and others 2003) and the Mississippi State Dept. of Health, U.S.A., respectively. Stock cultures were maintained at –65 °C in tryptic soy broth containing 10% glycerol. The cultures were thawed and reactivated by subculture in tryptic soy broth. Reactivated cells were stored on a tryptic soy agar slant. The strains were separately cultured in tryptic soy broth (Becton Dickinson, Sparks, Md., U.S.A.) with shaking at 150 rpm for 16 h at 37 °C (Incubator Shaker, New Brunswick, N.J., U.S.A.). After incubation, the bacterial cultures were added to sterilized, filtered, and pasteurized red muscadine juice to create a population of about 6.5-log CFU/mL (Lekkas and others 2006). The mixture was incubated at 37 °C, and the viable cells were recorded every 30 min, for up to 120 min. Tryptic soy broth (pH 3.2) acidified with hydrochloric acid was used as a control. Viable *C. sakazakii* were enumerated on tryptic soy agar (TSA) plates (Becton Dickinson) after incubation at 37 °C for 18 to 24 h. To see any recovery of cells after 2 h incubation, inoculated samples (100 μL) were transferred to TSB every 24 h, up to 72 h and then incubated on TSA for 24 h.

Antibacterial activity of synthetic organic acids found in red muscadine juice

Individual synthetic acid solutions at the concentrations found in red muscadine juice (malic acid, 1.27 mg/mL; tartaric acid, 3.37 mg/mL; tannic acid, 1.71 mg/mL) and their mixture were first prepared in sterile deionized water, and fructose and glucose were then added to make 16 °Brix for each solution. Their antimicrobial activities were measured as described above.

Statistical analysis

Data for pH, total phenolics, phenolic compounds, and organic acids of the juice samples were analyzed using a one-way factorial, while a 2-way factorial arrangement (treatments × time) was used for measurement of antimicrobial activity. Data obtained from at least 3 replications in a completely randomized design (except soluble solids) were analyzed by PROC general linear model, and means separated by Fisher's protected least significant difference ($P < 0.05$) using SAS 9.1 (SAS Inst., Cary, N.C., U.S.A.).

Results

Soluble solids, pH, total phenolics, organic, and polyphenols in red muscadine and commercial baby juices

Since organic substances in fruit beverages could be responsible for their antibacterial activity, total soluble solids (°Brix), pH, organic and phenolic acids, and major phenolic compounds in red muscadine and commercial baby juices were measured by using HPLC and the results were shown in Table 1 and 2. While the soluble solids of red muscadine juices was approximately 16 °Brix, commercial baby juices showed varied (13.4 to 17.4 °Brix) depending on the products (Table 1). Overall, red muscadine juices were higher ($P < 0.05$) in tartaric acid than commercial baby juices (Table 1). Tannic acid was the major phenolic acid in all red muscadine juices, while commercial baby juices did not contain tannic acid (Table 1). Red muscadine juices and commercial mixed berry juice showed the highest content of total phenolics, followed by commercial apple grape juice and then white grape juice (Table 1). Among the major phenolic compounds detected, ellagic acid (0.3 mg/mL) was 7 to 11 times higher in red muscadine juices than in commercial baby juices (Table 2). Regardless of treatment of red muscadine juices, gallic acid, catechin, epicatechin, ellagic acid, and resveratrol did not differ in their concentration.

Antimicrobial activity of red muscadine and commercial baby juices on *C. sakazakii*

The antimicrobial activity of red muscadine and commercial juices against 2 *C. sakazakii strains* was measured by the aerobic plate count method on TSA. Within 2 h, both strains of *C. sakazakii* were reduced to nondetectable levels in all red muscadine juices (Figure 1A and 1B). Even though the decrease in viable *C. sakazakii* cells showed a lag trend in the first 0.5 h, there was about a 6-log reduction in cell number in 1.5 h, regardless of processing types of red muscadine juice (Figure 1A and 1B). However, commercial baby juices were not inhibitory on the 2 *C. sakazakii* strains: there was less than 1.0-log reduction of *C. sakazakii* within 2 h (Figure 1C and 1D). Among the 3 commercial baby juices, mixed berry juice demonstrated slightly better antimicrobial effect than apple and grape juices. There were no differences in susceptibility between *C. sakazakii* Fec39 and MSDH to either red muscadine or commercial baby juices.

Anti-*C. sakazakii* activity of synthetic acids found in red muscadine juice

Anti-*C. sakazakii* activity was determined in tartaric (3.37 mg/mL), malic (1.27 mg/mL), tannic acid (1.71 mg/mL), and the mixture of these compounds (MXA) with fructose and glucose mixture (16 °Brix), equivalent to concentration in red muscadine juice (Table 1). The pH of all synthetic acid solutions was adjusted to 3.2. The initial number of cells inoculated in the solution was approximately 7.5-log CFU/mL at 37 °C (Figure 2A and 2B). The mixed acid, MXA solution (Figure 2) was not as effective antimicrobial as any of the juice treatments (Figure 2). Among synthetic acids, tannic acid showed highest anti-*C. sakazakii* activity on both strains. Polyphenol fractions (polyphenols and pigments) did not show any anti-*C. sakazakii* activity (data not shown). This result implies that tannic acid in muscadine juices is the key component for antibacterial activity.

Discussion

The red muscadine juice contained tartaric (3.4 mg/mL), malic (1.27 mg/mL), tannic acids (1.71 mg/mL) as polar compounds (Table 1) and gallic acid (0.2 mg/mL), catechin (0.54 mg/mL), epicatechin (0.54 mg/mL), ellagic acid (0.24 mg/mL), myricetin (0.01 mg/mL), resveratrol (0.04 mg/mL), and quercetin (0.01 mg/mL) as nonpolar compounds (Table 2). These organic components in red muscadine juice were also found in the water-soluble seed extracts of "Ison" (red) and "Carlos" (bronze) which contained tartaric (6.6 to 7.27 mg/mL), malic (1.33 to 1.87 mg/mL), tannic (4.3 to 7.1 mg/mL), ellagic (0.02 to 0.17 mg/mL), gallic acids (0.11 to 0.35 mg/mL), catechin (0.07 to 0.18 mg/mL), and epicatechin (0.05 to 0.1 mg/mL) (Kim and others 2008, 2009a, 2009b).

Regardless of treatment, the red muscadine juices inactivated more than 5-log cells of 2 *C. sakazakii* strains within 1.5 h. Previous studies showed that water-soluble muscadine seed extracts inactivated 5-log cells of 2 strains of *C. sakazakii* (Kim and others 2009b). When whole muscadine grapes are heated, crushed and pressed, water-soluble antimicrobial components from muscadine

Table 1 — Soluble solids, pH, total phenolics, organic and phenolic acids in red muscadine and commercial baby juices.

				(mg/mL)			
Juice type	Treatment	Soluble solids (°Brix)	pH	Total phenolics	Tartaric acid	Malic acid	Tannic acid
Red muscadine juice	Fresh	16.3	3.16b[a]	338.4a	3.37b	1.27a	1.71a
	Filtration	16.2	3.16b	319.4a	4.27a	1.60a	1.90a
	Sterilization	15.4	3.19ab	321.6a	4.48a	1.78a	1.83a
	Pasteurization	16.0	3.22a	322.9a	4.65a	1.48a	1.41b
Commercial baby juices	Apple grape	13.4	3.47a	210.7b	0.67d	4.45a	ND[b]
	Mixed berry	15.8	3.29b	347.7a	0.99d	3.62b	ND
	White grape	17.4	3.51a	176.7c	1.67c	2.87c	ND

[a]Means of muscadine or baby juices followed by a different letter differ ($P < 0.05$).
[b]ND = not detected.

Table 2 — Major phenolic compounds in red muscadine and commercial baby juices.

		(mg/100 mL)						
Juice	Treatment	Gallic acid	Catechin	Epicatechin	Ellagic acid	Myricetin	Resveratrol	Quercetin
Red muscadine juice	Fresh	21.6*a*[a]	54.0a	53.7a	24.4a	1.1a	3.8a	0.9b
	Filtration	21.1a	53.4a	50.7a	29.1a	0.5b	4.6a	1.3ab
	Sterilization	20.2ab	43.0b	47.6a	25.5a	1.4a	4.4a	0.6b
	Pasteurization	18.0b	43.2b	43.9a	24.0a	0.9ab	4.4a	1.7a
Commercial baby juice	Apple grape	17.7b	32.5a	46.5a	2.60b	0.56a	5.05ab	ND[b]
	Mixed berry	19.7ab	29.7a	46.2a	3.17b	0.70a	2.10b	ND
	White grape	22.8a	26.7a	14.5c	2.20b	0.63a	6.93a	0.4a

[a]Means of muscadine or baby juices followed by a different letter differ ($P < 0.05$).
[b]ND = not detected.

seed might be released into their juice, which might explain the antimicrobial activity of juices. However, The hot Ison seed extracts reduced the number (approximately 6-log CFU/mL) of cells of *C. sakazakii* Fec39 and *C. sakazakii* MSDH to nearly undetectable levels within 0.5 h (Kim and others 2009b), whereas red muscadine juice (Noble) inactivated less than 2-log cells in the same time period. Low antimicrobial activities of the juices as compared to seed extracts could be due to the lower concentration of malic, tartaric, and tannic acids in red muscadine juice than in seed extracts. In a previous study, we demonstrated that only the mixture of polar compounds such as malic, tartaric, and tannic acids showed antimicrobial activity on 3 strains of *Escherichia coli* O157:H7 (Kim and others 2009a). While the mixture of artificial sugars and acids inactivated only 3.8- and 3.9-log cells on *C. sakazakii* Fec39 and MSDH within 2 h, respectively, red muscadine juice inactivated all inoculated cells (6.5-log CFU/mL) in the same time period. Our previous findings (Kim and others 2009a) also showed that the presence of natural acids and sugars in muscadine juice showed much higher anti-*E. coli* O157:H7 activity than their synthetic mixture. The presence of sugars in synthetic acid solution retarded their antimicrobial activity on *E. coli* O157:H7 and *C. sakazakii*. Rowbury and Goodson (1998) explained a pH homeostatic mechanism where glucose rapidly induced marked acid tolerance in Gram negative bacteria; which showed less acid damage to DNA and

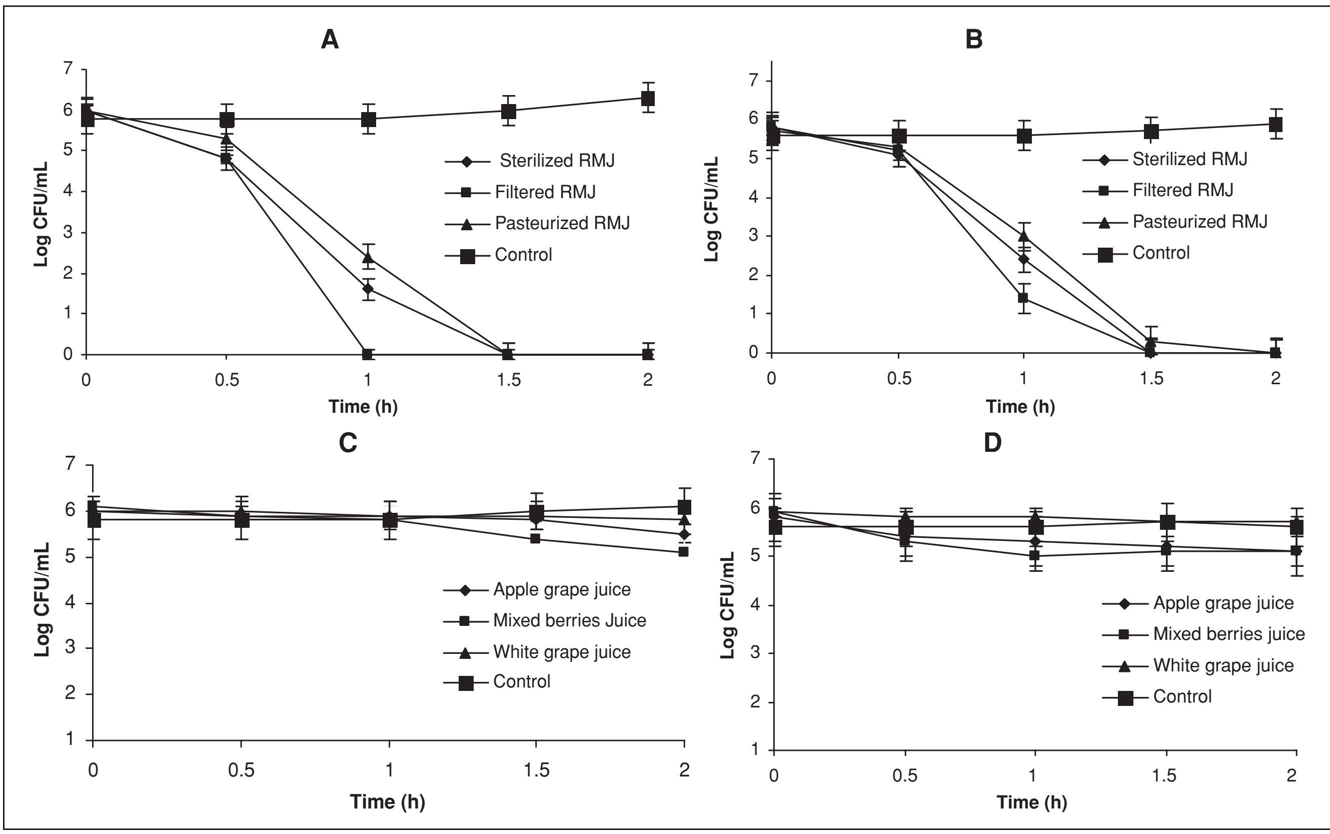

Figure 1—Viable cells of *Cronobacter sakazakii* Fec39 (A, C) and *C. sakazakii* MSDH (B, D) in red muscadine juices (RMJ) (A, B) and commercial baby juices (C, D). Control was tryptic soy broth adjusted to pH 3.2.

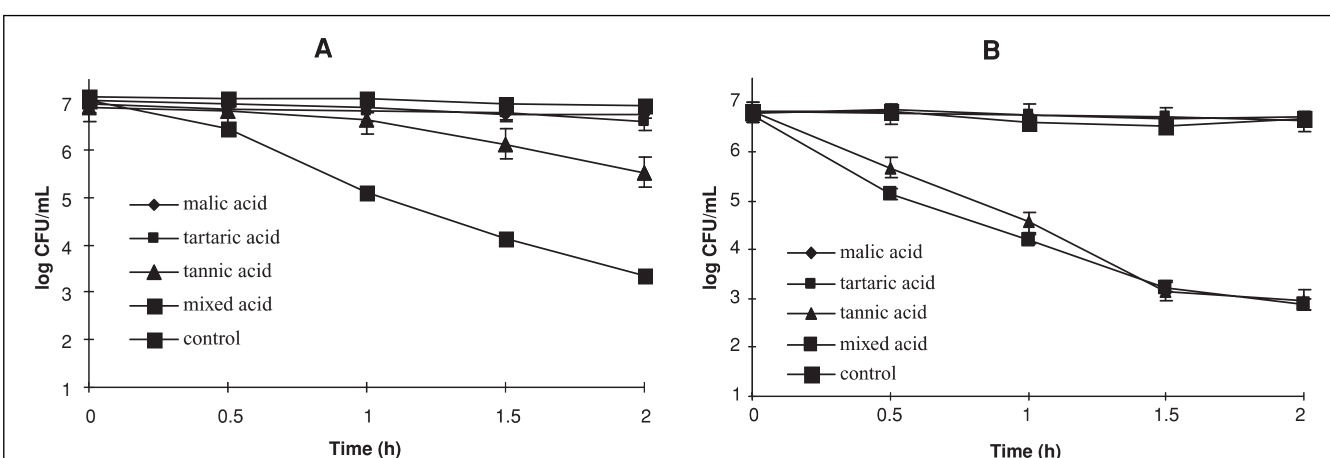

Figure 2—Inhibition of *Cronobacter sakazakii* Fec39 (A) and *C. sakazakii* MSDH (B) by individual synthetic acids and their mixture in a sugar (16 Brix with glucose and fructose) solution. Control was tryptic soy broth adjusted to pH 3.2.

ß-galactosidase. *Cronobacter sakazakii* have been reported as a moderately acid resistant member of the *Enterobacteriacea.* Tryptic soy broth (pH 3.2) acidified with hydrochloric acid did not show any reduction of cells (Figure 1). Edelson-Mammel and others (2006) observed that 10 of 12 strains of *C. sakazakii* showed less than a 0.5-log reduction in tryptic soy broth at pH 3.5, adjusted hydrochloric acid. Predominant organic acids (citric and malic acids) in apple and strawberry juices with low pH values (3.6 to 3.9) did not inactivate low inoculum numbers (approximately 1.5-log CFU/mL) of *C. sakazakii* (Kim and Beuchat 2005; Lin and Beuchat 2007). All the commercial baby juices tested in this study contained almost the same levels of organic acids and phenolic acids as red muscadine juices, but did not have tannic acid. Commercial baby juices that did not contain tannic acid showed much less anti-*C. sakazakii* activity, suggesting that tannic acid is the key component responsible for the anti-*C. sakazakii* activity of the muscadine juices. Data showed that regardless of their pH (3.2 to 3.8) or processing method (sterilization, filtration, and so on), tannic acid (1.7 mg/mL) was the most active antimicrobial compound among the 3 polar compounds, showing approximately 4-log reduction on *C. sakazakii* within 2 h (Figure 2). Previous studies have reported the antimicrobial activity of tannic acid against *Aeromonas hydrophila* and *Aeromonas sobria* (Chung and others 1998), *E. coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Staphylococcus aureus* (Chung and others 1993), *Helicobacter pylori* (Funatogawa and others 2004), *Cytophaga columnaris* (Zhao and others 1997), HIV viruses (Mizumo and others 1992), and influenza virus (Green 1948; Carson and Frisch 1953). The antimicrobial mechanism of tannic acid seems to be totally different from the antimicrobial action of organic acids. It may work like a siderophore to chelate essential iron from the medium and make iron unavailable to the microorganisms (Chung and others 1998).

Conclusions

In conclusion, red muscadine juice, a rich source of phenolic acids and organic acids, including the important tannic acid, showed strong antimicrobial action against *C. sakazakii.* This result suggests that red muscadine juice may have the potential to be used in baby foods as a natural *C. sakazakii* inhibitor.

Acknowledgments

Approved for publication as Journal Article Nr J-11694 of the Mississippi Agricultural and Forestry Experiment Station, Mississippi State Univ. This research was supported in part by the Mississippi Agricultural and Forestry Experiment Station Project Nr MIS-371272 and by USDA-ARS Grant Nr 58-6402-6-075.

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